CHAPTER IV
RESULTS

1. Cytotoxicity and subcytotoxic concentration

To determine the effect of each compound on the mitochondrial reductase activity of the target cells 7 compounds from *C. nutans* and 3 compounds from *A. paniculata* were examined for cytotoxicity in A549 cells using the MTT assay. The confluent A549 cell monolayer growing in medium containing the compounds with different concentration was observed for cell death and cell viability (%). The result was compared with cell control growing in medium without compound and CC_{50} was calculated. Subcytotoxic concentration of each compound was the concentration showing no sign of cytotoxicity. Table 2 shows name or chemical formula, CC_{50} and subcytotoxic concentration of each compound used in further study of anti-DV2-16681 activity.

Table 2 50% cytotoxicity and subtoxic concentration of the studied compounds.

Name	Name or chemical formula	MW	CC_{50} (μM)	Subcytotoxic conc. (μM)
11(3)	$C_{55}H_{70}N_4O_7Mg$	922.5	54	14
136A	(*C. nutans*)		73	46
23B	$C_{55}H_{70}N_4O_7Mg$	922.5	616	369
23C	(*C. nutans*)		203	122
136C	$C_{55}H_{72}N_4O_7$ (*C. nutans*)	900	55	14
136D	$C_{53}H_{70}N_4O_5$ (*C. nutans*)	842	59	30
136B	$C_{55}H_{74}N_4O_5$ (*C. nutans*)	870	29	15
SS1	Andrographolide (*A. paniculata*)	350.209	94	57

Table 2 50% cytotoxicity and subtoxic concentration of the studied compounds (Cont.).

Name	Name or chemical formula	MW	CC_{50} (μM)	Subcytotoxic conc. (μM)
SS2	14-deoxy-11,12-didehydroandrographolide (*A. paniculata*)	332.199	30	12
SS3	3, 19- isopropylidene andrographolide (*A. paniculata*)	390.241	85	51

2. Anti-DV activity of compounds from *C. nutans* and *A. paniculata*.

2.1 Screening inhibitory effect of compounds from *C. nutans* and *A. paniculata* on DV2 infection

The preliminary screenings among the ten compounds were tested for inhibitory effect to DV2 strain16681 infection. The subtoxic concentration of each compounds were incubated with DV2 at 0.01 MOI at room temperature for 1 h. Then each mixture was adsorbed to the confluent A549 cells. After virus adsorption, the infected cells were cultured in medium containing the subtoxic concentration of each compound. 200 μM of ribavirin was used as a positive control. After 72 h of infection, viral RNA, representation of viral replication, was determined. Total RNA was then extracted from the infected cell and converted to cDNA that was used for amplification with DV2 specific primers by RT-PCR. The results showed that most compounds did not effect on DV infection because of viral RNA was positive by RT-PCR (Figure18). The positive detections were corresponded to result from detection of infectious virus using IF technique at 7 days of infection. There were two compounds from *C. nutans*, phaeophorbide a (136B) and 13^2-hydroxy-(13^2-*S*)-chlorophyll b (11(3)) and one compound from *A. paniculata*, 14-deoxy-11,12-didehydroandrographolide (SS2) showed positive result in this screening inhibitory study that viral RNA as well as fluorescent infected cell were negative (Figure 19). This result indicated that these compounds had some anti-viral activities and were used in further study at the level of pre-entry and post-entry step.

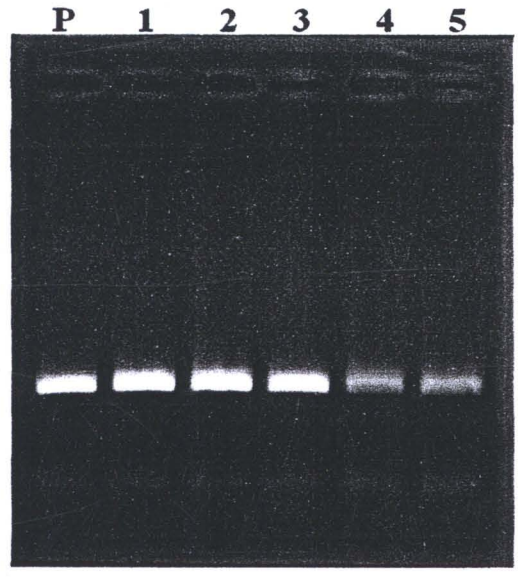

Figure 18 Screening of anti-DV2 activities. Lack of inhibition DV2 infectivity and viral RNA synthesis. Lane P; positive control, Lane 1; treated 136A, Lane 2; treated 136C, Lane 3; treated 136D, Lane 4; treated SS1, Lane 5; treated SS3.

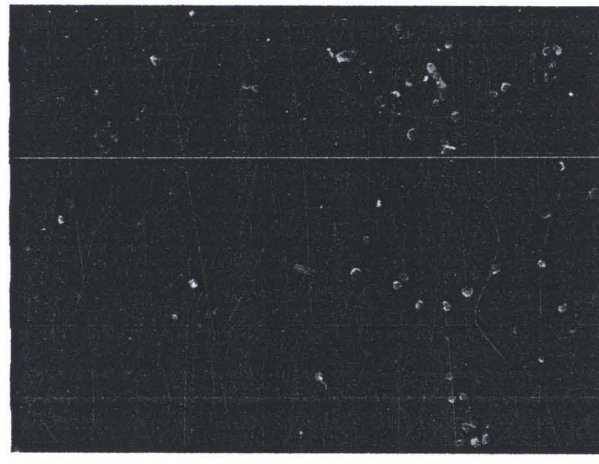

Figure 19 The fluorescent infected cells detected by IF technique at 7 days post-infection.

2.2 Anti-DV2 activity in pre-entry

All three compounds (136B, 11(3) and SS2) were further investigated for anti-viral activity in the step of pre-infection. Subcytotoxic concentration of each compound was co-incubated with DV2 at 0.01 MOI at room temperature for 1 h before adsorption. This virus-compound mixtured was used to infect the confluent

A549 cells. Dextran sulfate sodium salt was mixed with virus and used as a positive control. After virus adsorption, the mixture was aspirated and the infected cells were incubated in 2%FBS-RPMI medium, and then incubated at 37°C for 72 h. After 72 h of infection, total RNA was extracted from infected cell and and used for viral RNA detection by RT-PCR. The results showed inhibitory effect of 11(3) and SS2 at the pre-entry step. Figure 20 demonstrated no amplified PCR product from RNA sample of A549 cells infected with the mixture of virus-compound (SS2 and 11(3)), whereas no inhibitory effect was observed with compound 136B. This finding was confirmed by IF technique that performed in infected cells incubated for 7 days after infection (Figure 21).

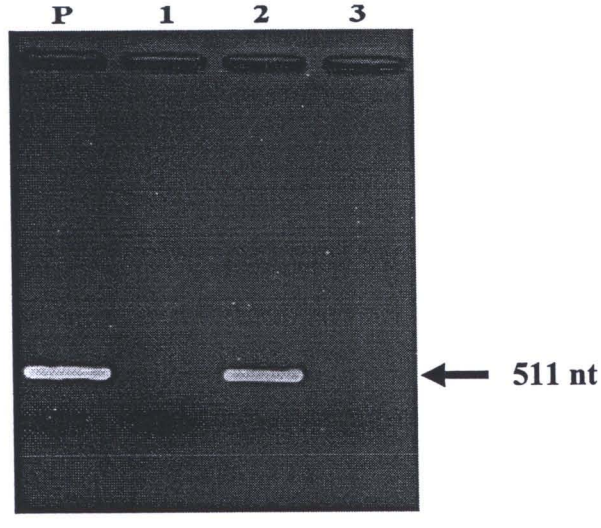

Figure 20 Anti-DV2 activity in pre-entry step. Inhibition of DV2 infectivity and viral RNA synthesis by SS2 and 11(3). Lane P; positive control, Lane 1; treated SS2, Lane 2; treated 136B, Lane 1; treated 11(3).

Figure 21 The tested cells without fluorescent signal detected by IF technique at 7 days post-infection.

2.2.1 Anti-DV activity in adsorption step

The effect of 11(3) and SS2 on anti-DV infection in adsorption step being in pre-entry step of viral replication cycle was confirmed. The experimental approaches were determined the amount of bound viruses in the presence of compound. The inhibition of virus adsorption by the 11(3) and SS2 were investigated after DV adsorption on A549 cells at 4°C in the presence of compound at subtoxic concentration.The infected cells were incubated in 2%FBS-RPMI medium at 37°C for 72 h. The amount of A549 cell-bound infectivity was inhibited as shown in Figure 22 and 23. Amplified PCR product was not detected in A549 cells treated with mixture of virus-compounds, whereas positive control showed the positive band as shown in Figure 22 at 7 days after incubation. The IF technique was also confirmed the negative result of the infected cells. This result suggested that 11(3) and SS2 compounds may have virucidal or modulation effect on virus particles that influent to the infectious process.

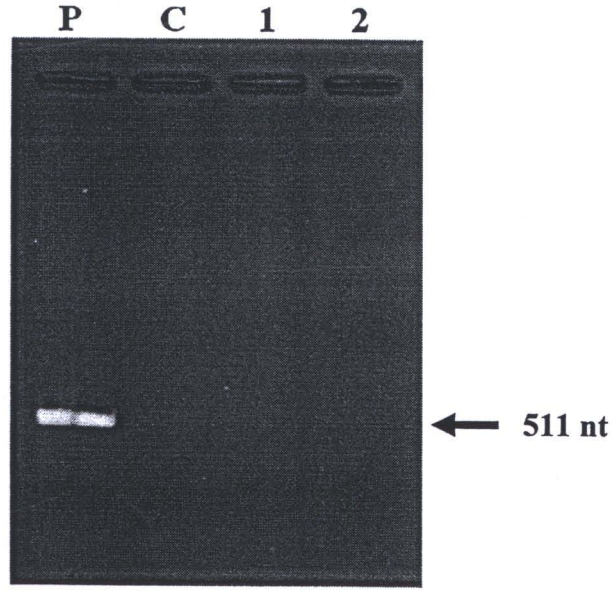

Figure 22 The effect of 11(3) and SS2 compounds on anti-DV infection in adsorption step shows no 511 nt PCR product. The PCR product bands were visualized in 1.5% agarose gel electrophoresis and stained with ethidium bromide. Lane P; positive control, Lane C; cell control, Lane 1; treated 11(3), Lane 2; treated SS2.

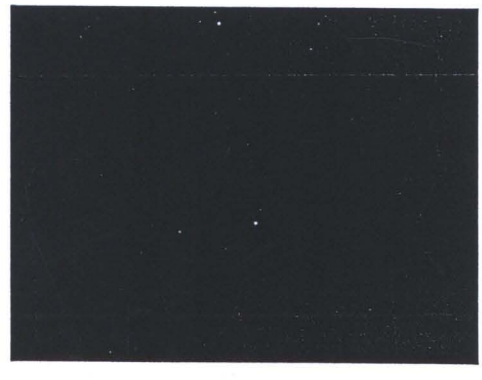

Figure 23 The effect of 11(3) and SS2 compounds on anti-DV infection in adsorption step show tested cells without fluorescent signal detected by IF technique at 7 days post-infection.

2.2.2 Pre-binding assay

The 11(3) or SS2 was added on the target cells for 1 h prior infection with DV to determine whether these compounds can inhibit viral entry through blocking receptors on the target cells. The experiment approached by treatment of confluent A459 cell

with 11(3) or SS2 before DV infection. Viral RNA in infected cells was also determined after 72 h incubation. The result showed no evidence of inhibition and the amplified PCR product were found in similar pattern of positive control (Figure 24). The result indicated that these compounds did not interact with or modify the target cells.

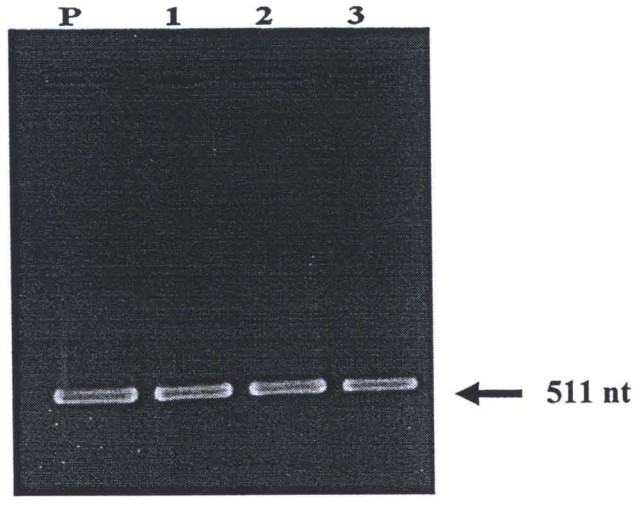

Figure 24 Lack of inhibitory effect of compounds on pre-binding assay. The RT-PCR product bands were visualized in 1.5% agarose gel electrophoresis and stained with ethidium bromide. Lane P; positive, Lane 1; treated SS2, Lanes 2; treated136B, Lane 3; treated 11(3).

2.2.3 Anti-internalization

To assess the possibility of compound on internalization step of DV replication cycle, DV was adsorbed on confluent A549 cells in the absence of compound at 4°C for 1 h, then cultures of infected cells were shifted the incubation to 37°C in the condition containing compounds for 1 h, after incubation in 2%FBS-RPMI medium at 37°C for 72 h, viral RNA in the infected cells was determined. Figure 25 showed the amplified PCR products that were found with similar pattern of viral infected control cells. Figure 26 also confirmed the positive result by immunofluorescenc of infected cell. The result suggested that these compounds (11(3) and SS2) were not affected to internalized process after adsorption of DV infection.

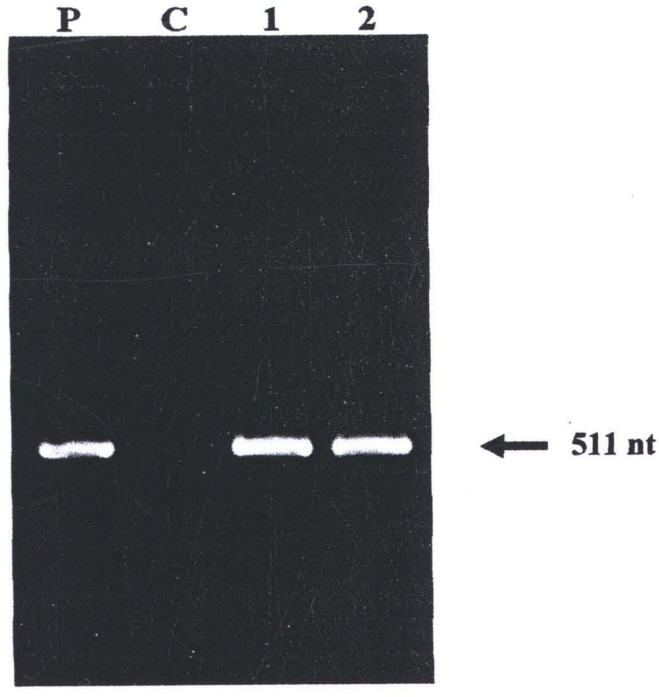

Figure 25 Lack of inhibitory effect of compounds on anti-internalization step. The RT-PCR product bands were visualized in 1.5% agarose gel electrophoresis and stained with ethidium bromide. Lane P; positive, Lane C = cell control, Lane 1; treated SS2, Lanes 2; treated 11(3).

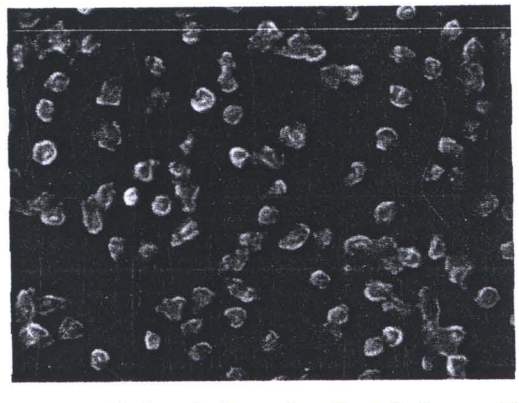

Figure 26 Immunofluoresce staining infected cell at 7 days of infection (positive).

2.3 Anti-DV2 post-entry

136B, 11(3) and SS2 were investigated for anti-DV infection at post-entry step to confirm the inhibitory effect. At the subtoxic concentration, they were added to confluent A549 target cells that were infected for 1 h with DV2 and the infected cell were maintained in medium with each compound for 72 h. Treatment of DV2 infected

cells with 11(3) and SS2 after viral entry did not effect on viral replication as shown in the Figure 27 The amplified PCR product of viral RNA was detected, whereas treatment DV2 infected cell with 136B inhibit DV replication in the target cell. This indicated that 136B had anti-DV activity at post-entry step.

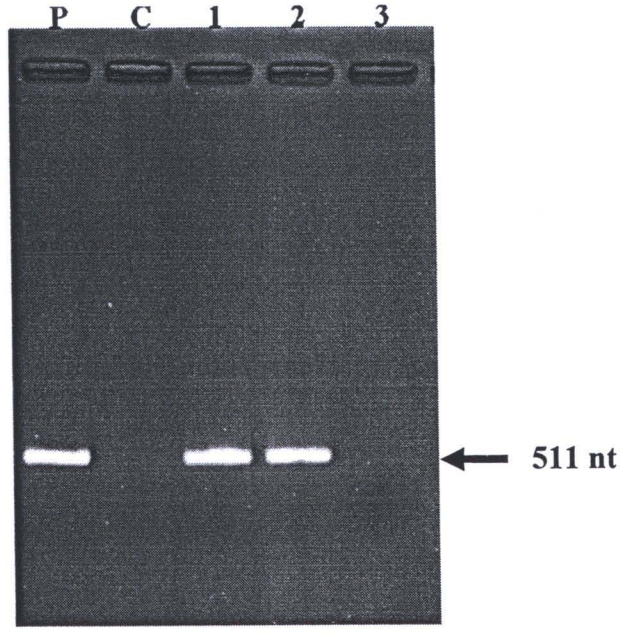

Figure 27 Anti-DV2 activities in post-entry of compounds by RT-PCR method. The RT-PCR product bands were visualized in 1.5% agarose gel electrophoresis and stained with ethidium bromide. Lane P; positive, Lane C = control (treated ribavirin), Lane 1; treated SS2, Lanes 2; treated 11(3), Lane 3; treated 136B.

3. Effect of compounds from *C. nutans* and *A. paniculata* on pro-inflammatory cytokine

3.1 DV-induced PGE_2 production

Recently the studies reported that DV induced COX-2 gene expression and PGE_2 production. To determine whether DV infection induces PGE_2, an inflammatory response, the confluent A549 cells were infected with mock or DV at MOI 1, 0.1 and 0.01 and then collected at 48 h post-infection. Total cell lysates were prepared and analyzed for PGE_2 concentration using ELISA method. In A549 cells, PGE_2 was constitutively produced with low level, whereas dose-dependently enhanced were

occurred by DV infection (Figure 28). PGE_2 concentration of the test was also prominently enhanced by DV2 infection at 48 h post infection when it was compared with control cells.

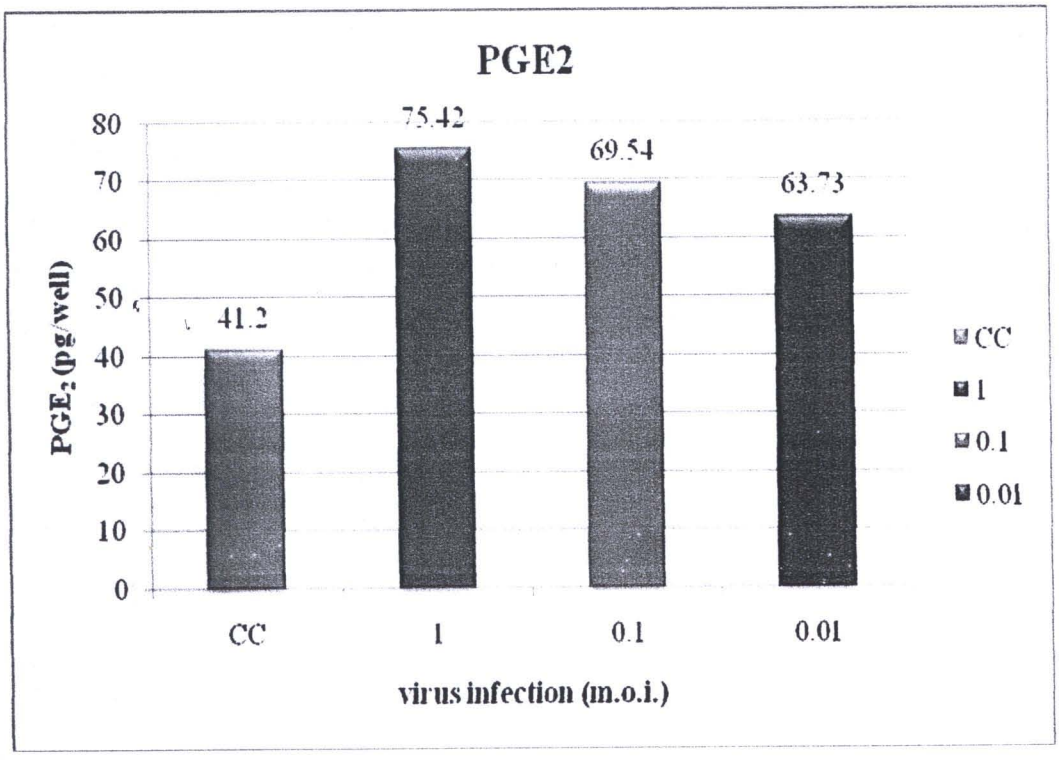

Figure 28 DV stimulated PGE_2 production in A549 cell. PGE_2 level at various concentrations in mock- or DV-infected cells.

3.2 Effect of compounds from *C. nutans* and *A. paniculata* on PGE_2 production

C. nutans and *A. paniculata* are the medical Thai herbs, both possessing immune-modulation functions. This study aimed to characterize the capability of these compounds to induce PGE_2 production in A549 cells using ELISA method. The confluent A549 cells were treated with each compound at the subtoxic concentration, and then collected at 48 h post-incubation. The result showed that all compounds stimulated PGE_2 production in A549 cells when compared with control cells (Figure 29).

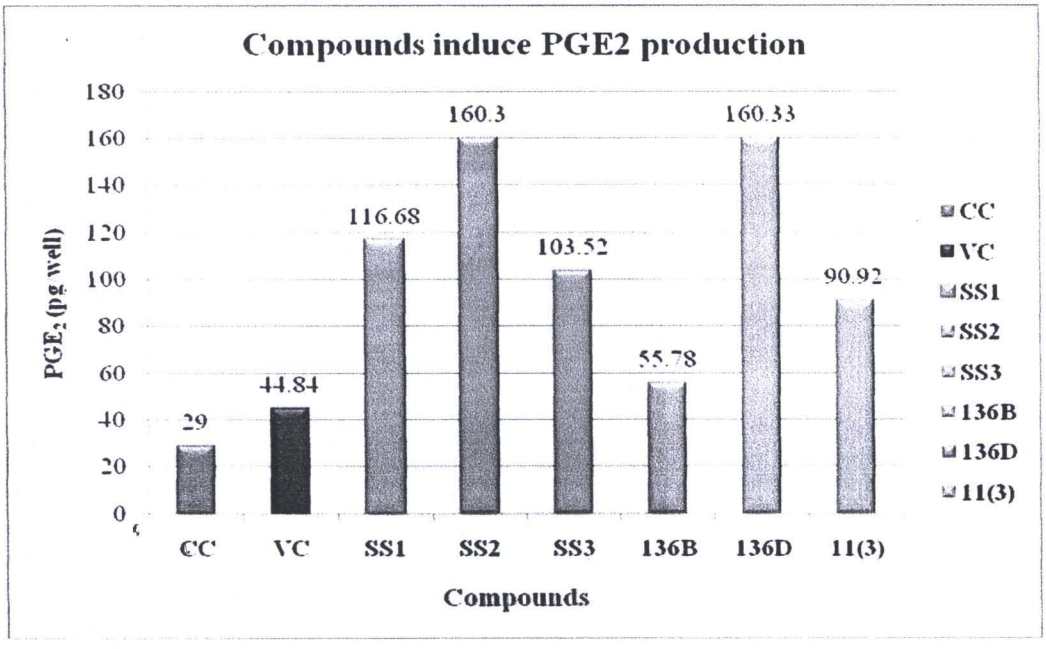

Figure 29 All compounds from *C. nutans* and *A. paniculata* stimulated PGE_2 production in A549 cells.

3.3 Effect of compounds from *C. nutans* and *A. paniculata* on virus-induced PGE_2 during post-infection

To examine the immunomodulation effect of compounds from *C. nutans* and *A. paniculata* on DV infected cells, the confluent A549 cells were infection with DV at 0.01 MOI or mock. After viral adsorption for 1 h, the infected cells were treated or incubated with subcytotoxic concentration of these compounds. The cell lysates were analyzed for PGE_2 production at 48 h post-infection by ELISA method. Fold change of PGE_2 level was compared with cell control, dengue virus-infected A549 cells without treatment (Figure 30). The results showed that PGE_2 production was enhanced in DV infected cells that were treated with these compounds (SS1, SS2, SS3, 136A, 136C, 136D, 11(3)) excepting 136B showed the PGE_2 lower than DV2-infected cell.

All three compounds isolated from *C. nutans* (136B and 11(3)) and *A. paniculata* (SS2) that contained potent anti-DV2 activity also affected to PGE_2 production in DV2-infected cells (Figure 31 A-C). PGE_2 level was slightly decreased SS2 and 11(3) treated DV infected cell, whereas in 136B treated DV infected cell the PGE_2 level showed similar to control cells. By RT-PCR, COX-2 gene expression was

evaluated to confirm immune modulation effect of compound 136B. The result showed that decreasing of COX-2 gene expression correlated well with the PGE_2 production (Figure 31). This result showed that the DV-induced COX-2 expression in A549 cells was apparently inhibited by 136B (Figure 32).

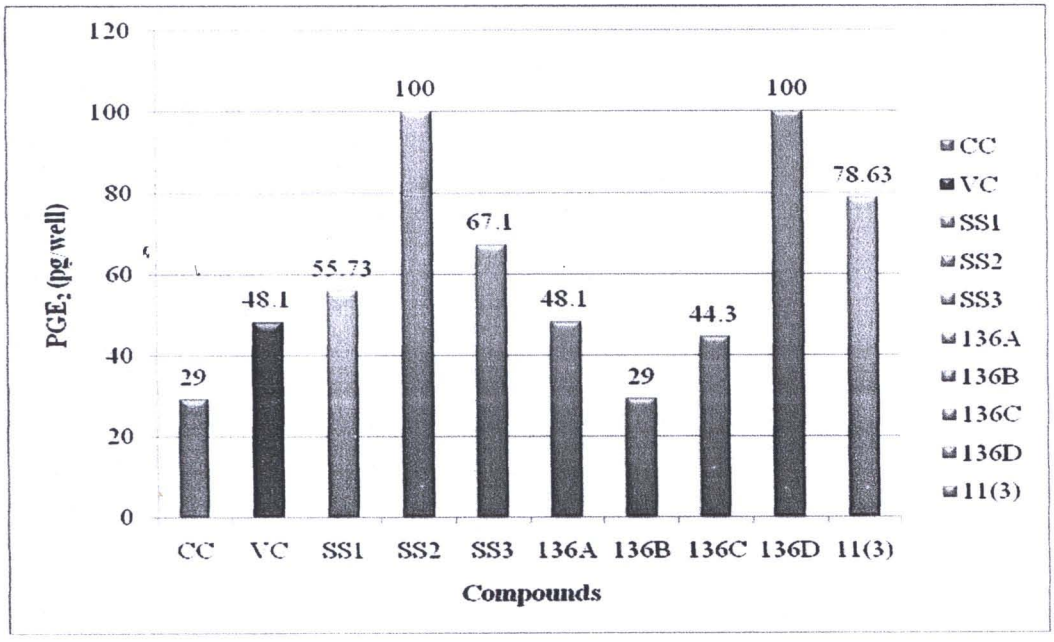

Figure 30 COX-2 expressions in infected A549 cells treated with compounds from *C. nutans and A. paniculata.*

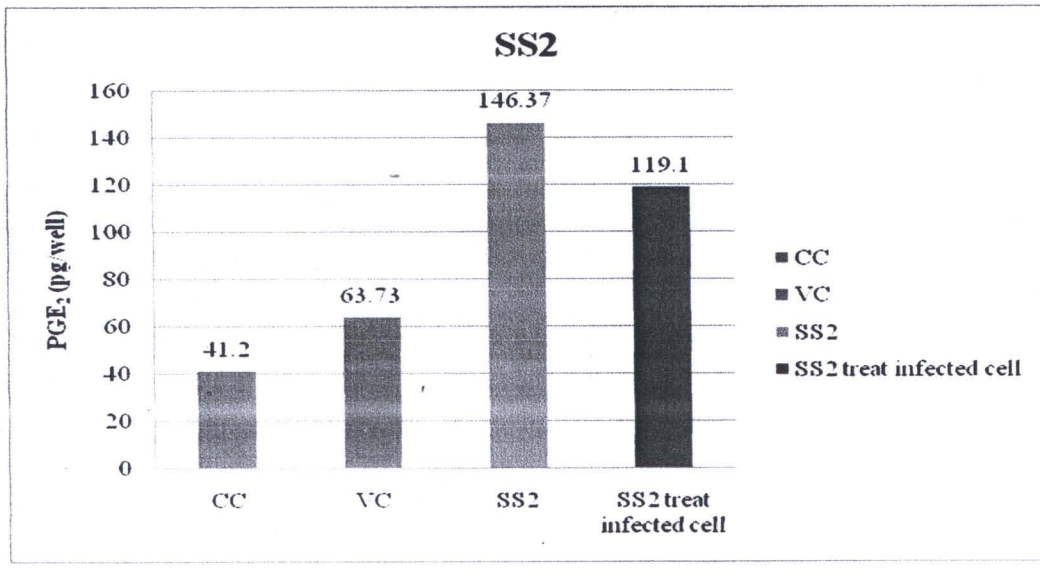

Figure 31 A The amount of PGE_2 produced by SS2 compound treated cells was mostly higher than those by control cells and DV2-infected cells.

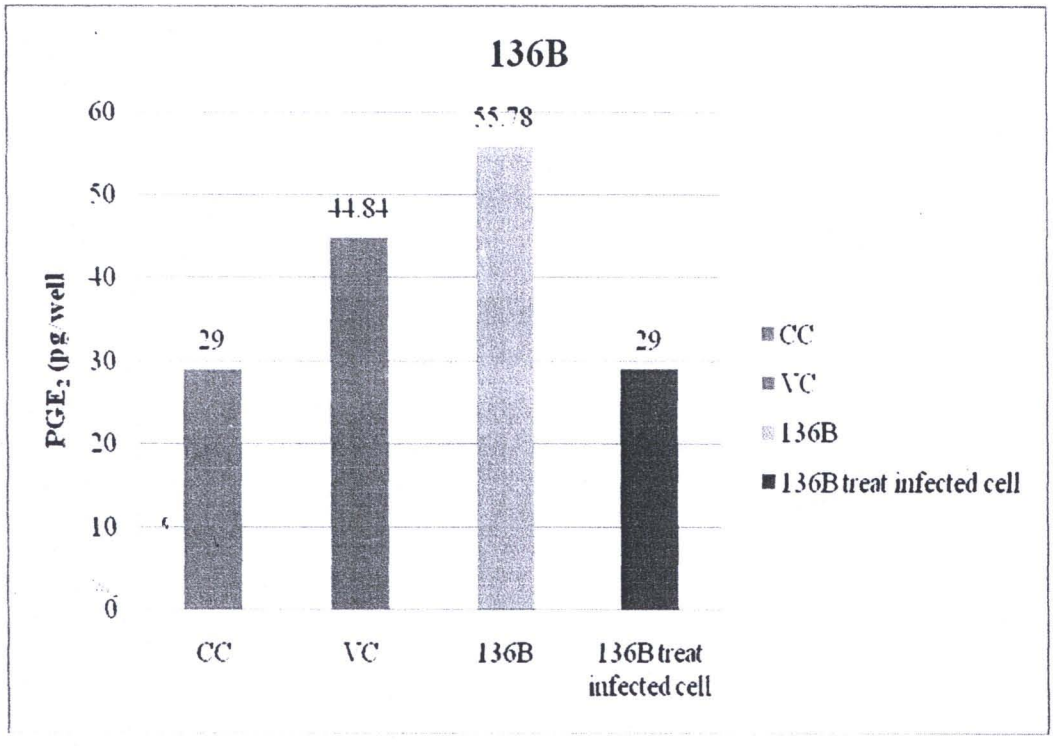

Figure 31 B Show that DV2-induced PGE_2 production was inhibited by 136B compound from *C. nutans.*

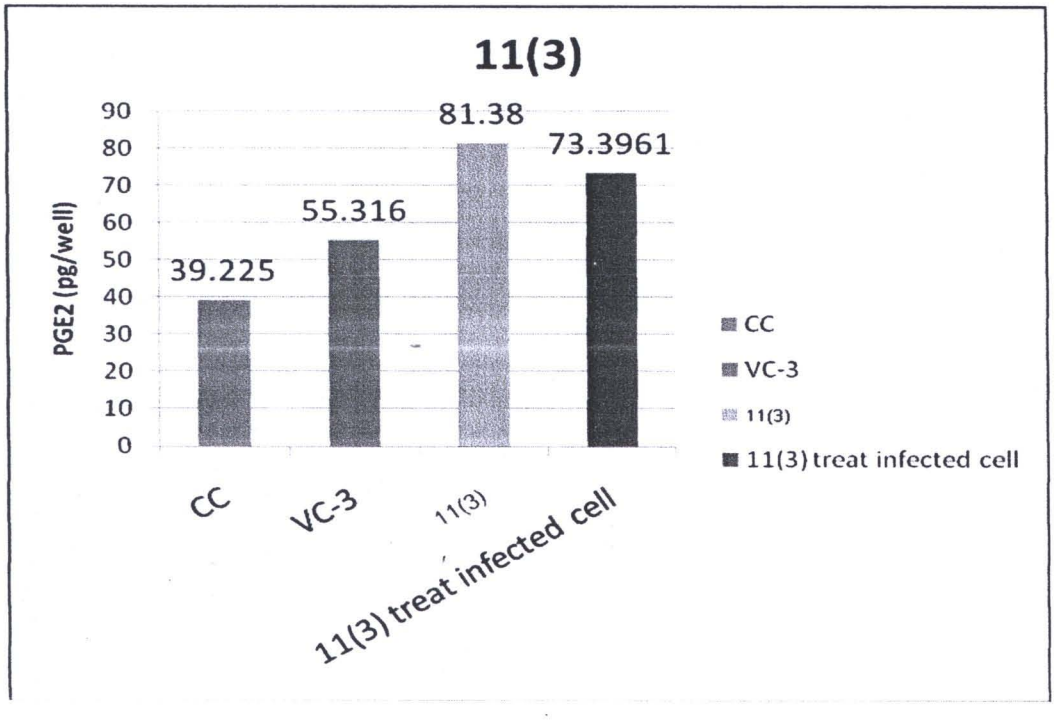

Figure 31 C The amount of PGE_2 produced by 11(3) compound treated cells was mostly higher than those by control cells and DV2-infected cells.

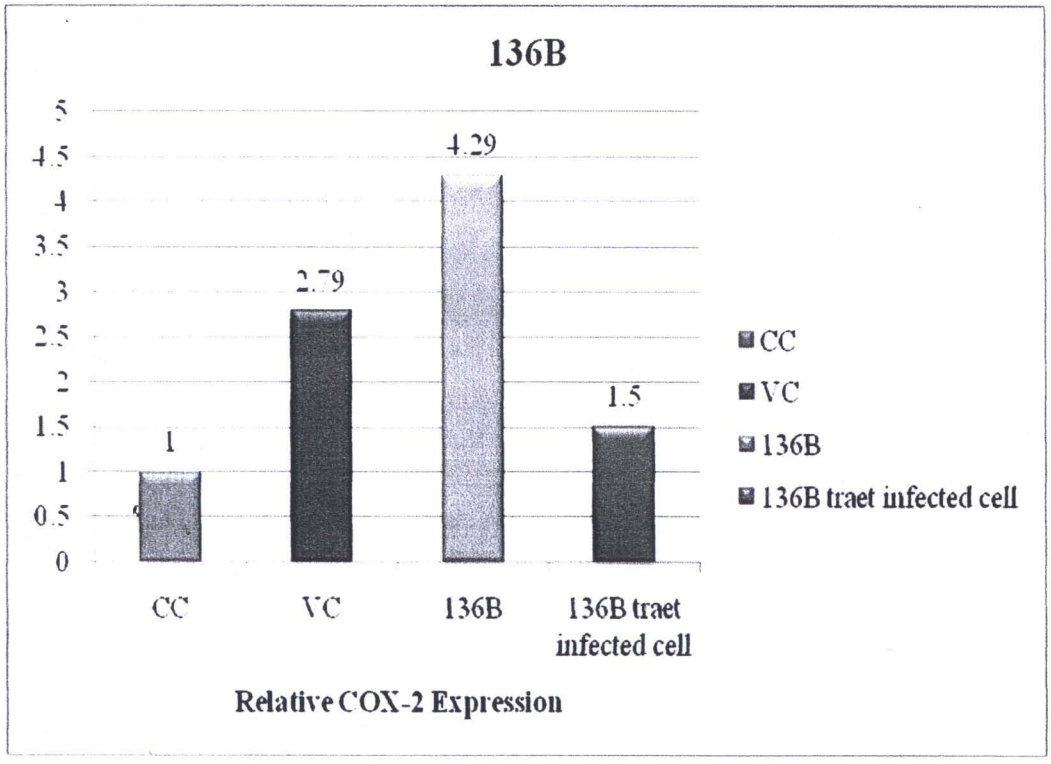

Figure 32 DV-induced COX-2 expression in A549 cells was apparently inhibited by 136B.